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EFFECTS OF STRONTIUM ON THE EMBRYONIC DEVELOPMENT OF *APLYSIA CALIFORNICA*

JOSEPH P. BIDWELL,* JOHN A. PAIGE,* AND ALAN M. KUZIRIAN

* *Howard Hughes Medical Institute, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, and Section on Neural Systems, Laboratory of Biophysics, NINCDS-NIH, Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

ABSTRACT

Strontium is required in defined media for the development of normal shells and statoliths in the opisthobranch gastropod, *Aplysia californica* Cooper, 1863. Embryos grown in artificial seawater without strontium chloride become larvae with normally appearing soft tissue but with deformed shells and statocysts that lack the statolith granule. Shell and statolith formation is sensitive to concentration differences as small as one part per million strontium in the artificial seawater. Pulse experiments indicate a critical period of exposure for the requirement beginning at Day 4, (72 h after oviposition) and lasting no longer than 24 h. Animals exposed to artificial seawater containing 8 parts per million strontium only during this critical period develop normally. Conversely, animals immersed in artificial seawater lacking strontium during this same period, but exposed to the element during the rest of the 10-day embryonic phase, exhibit anomalous shells and statocysts. The effects are only marginally reversible if strontium is presented after Day 5. Strontium is also essential for the normal development of locomotory behavior. Animals that have been deprived of strontium swim erratically, spinning in tight circles. The pathogenesis of this behavioral disorder is explained by the lack of strontium during embryonic development which causes the major structural anomalies at the larval stage. The absence of the statolith results in a defective organ of balance which in turn causes the severe behavioral symptoms. A similar strontium effect in other molluscs, including *Hermissenda crassicornis* (Eschscholtz, 1831), is also documented.

INTRODUCTION

The presence of strontium in the molluscan shell may serve as a record of past and present-day environments (Nelson, 1962; Rucker and Valentine, 1966; Lee and Wilson, 1969), as an indicator of the physiological state of the animal (Dodd, 1965), and as a clue to evolutionary relationships (Lowenstam, 1964). Before the significance of this element's presence can be fully interpreted, however, it will be necessary to disentangle the effects of the various factors that control the strontium content of skeletal carbonates, *i.e.*, water chemistry, skeletal mineralogy, physiology, and environment (Dodd, 1967). The amount of strontium in bivalve and gastropod shells is among the lowest of invertebrate mineralized tissues, suggesting that these animals have an exceptional ability to discriminate against this element (Dodd, 1967). A fundamental question then arises: is strontium required by any molluscan form, and if so, what is its specific biological role?

In the course of developing a simple artificial seawater capable of supporting growth and development of the opisthobranch mollusc, *Aplysia californica* Cooper, 1863, we

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found that the presence of strontium is essential during embryonic development for normal shell and statolith formation. The statocysts, sense organs of spatial orientation, are the earliest neural structures to develop (Kandel, 1979), appearing when the embryo reaches the 300-cell stage. At this time the shell-gland has invaginated to form a narrow pit and soon after everts to form the shell cap which secretes the shell (Saunders and Poole, 1910). Embryonic development in *Aplysia* spp. occurs within the protection of an egg mass, deposited after fertilization by the hermaphroditic adult. The mass is composed of a long, thin gelatinous cordon, which surrounds transparent capsules, each containing from 18 to 20 fertilized eggs. Development is complete 8–12 days after fertilization at 20°C when the free-swimming, planktotrophic veligers emerge from the strand (Kandel, 1979). We report here the effects of varying the strontium concentration in artificial seawater on the embryonic development of *A. californica,* and describe the results of experiments designed to ascertain the critical period of exposure. Results of preliminary experiments with the nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) are also presented.

Materials and Methods

The basal medium used reflects the concentrations and relative proportions of the major constituents of natural seawater (Brewer, 1975), consisting of, in grams/liter, 23.88 NaCl; 10.68, $MgCl_2 \cdot 6H_2O$; 4.01, Na_2SO_4; 1.51, $CaCl_2 \cdot 2H_2O$; 0.725, KCl; 0.196, $NaHCO_3$; 0.086, NaBr (pH 8.10 and salinity 34‰). This artificial seawater was prepared from concentrated stock solutions of each reagent grade constituent dissolved in deionized water. The magnesium and calcium stock solutions were standardized by Mohr titration (Skoog and West, 1965). Strontium contamination of reagents, as listed by lot analysis (Fisher), ranged from 0.0 to 0.1%. Subsequent flame atomic absorption measurements failed to detect this element in the basal medium. Strontium is a conservative element of natural seawater and is present at a concentration of $9.10 \cdot 10^{-5}$ *M* at a salinity of 35‰ (Brewer, 1975). We varied the concentration of strontium from 0.0 to $9.07 \cdot 10^{-5}$ *M* (approximately 0–8 ppm in 1 ppm increments) through additions of strontium chloride.

In one experiment we used the basal medium plus H_3BO_3 (25 ppm); $NaH_2PO_4 \cdot H_2O$ (1 ppm, PO_4^{-3}); NaF (1 ppm, F^-); $MnSO_4 \cdot H_2O$ (1 ppm, Mn^{+2}); $Na_2MoO_4 \cdot 2H_2O$ (0.7 ppm, MoO_4^{-2}); LiCl (0.2 ppm, Li^+); RbCl (0.1 ppm, Rb^+); $Al_2(SO_4)_3 \cdot 18H_2O$ (0.04 ppm, Al^{+3}); and KI (0.07 ppm, I^-). This is a modification of a medium described by Segedi and Kelley (1964), an early version of the commercial product Instant Ocean® (Aquarium Systems, Mentor, Ohio) (Bidwell and Spotte, 1985).

Egg masses were obtained from breeding pairs of adult *A. californica,* maintained in a flow-through natural seawater system at Marine Biological Laboratory, Woods Hole, Massachusetts, with a pH range of 7.8–8.2 and a salinity of 31 ± 1‰. Each egg mass was washed in basal medium, cut into strands 1 cm in length, and distributed into sterile, plastic petri dishes, 12–14 strands per dish, equivalent to 40,000–50,000 eggs. Each dish was filled with approximately 50 ml of the experimental medium (enough to cover the strands completely) and incubated at 21°C under continuous illumination. Replicates were prepared for all test solutions and the natural seawater control. All solutions were changed twice daily to prevent the possibility of mineral and oxygen depletion. Embryonic development was monitored daily through the capsule wall using both dissecting and compound microscopes. The time scale used to stage development designates 0 h as the time of oviposition.

For the pulse experiments egg strands were alternately exposed to artificial seawater containing 0 ppm and 8 ppm strontium. The first experiment was designed to show whether any period of the eight to ten day embryonic phase was more sensitive to strontium deprivation and whether the deficits induced by the lack of strontium were

reversible. This test began with 16 petri dishes, each containing 10 egg strands. Eight dishes contained basal medium and the remaining eight contained basal medium plus strontium. At the end of Day 1, 24 h after oviposition, five strands from Dish 1 containing basal medium, were transferred to a new dish containing artificial seawater plus strontium (Treatment 1), the remaining five strands served as a control. Likewise, at the end of Day 2, five strands from Dish 2, containing basal medium, were transferred to a new dish containing 8 ppm strontium (Treatment 2). Conversely, egg strands started in artificial seawater plus strontium were transferred to fresh basal medium in a like manner. Thus by the end of Day 8 the experiment included 32 petri dishes, 16 treatments, and their controls (we also ran a natural seawater treatment as a further control). Water was changed twice daily as in the dose response experiments. Figure 1 is a graphical representation of the 16 pulse treatments.

The second pulse experiment was undertaken to confirm the results of the first and to determine the length of the critical period, *i.e.*, we wanted to know whether a short pulse of strontium at the appropriate stage of embryonic development (as indicated by the first experiment) would result in larvae with normal mineralized tissue. This experiment consisted of 11 treatments including controls of basal medium, basal medium plus 8 ppm strontium, and natural seawater. Again, all test media were changed twice daily. Figure 2 represents the various pulse treatments in this experiment. Four treatments called for incubating the egg strands (ten per dish) in basal medium throughout the experiment except for a short pulse of artificial seawater containing 8 ppm strontium. Treatment A was pulsed with strontium for 24 h from the beginning of Day 4 (72 h after oviposition). Treatment B was pulsed for 48 h, from Day 4 to the beginning of Day 6. Treatment C was pulsed with strontium for 24 h from the beginning of Day 5. Treatment H followed the regimen as outlined for Treatment A except natural seawater was used in place of artificial seawater containing 8 ppm strontium.

Conversely, two other treatments required the strands to be incubated in artificial seawater with strontium and pulsed with basal medium at the beginning of Day 4, Treatment D for 24 h, and Treatment E for 48 h. Similarly the last two treatments, F and G, were incubated in natural seawater and pulsed with basal medium at the beginning of Day 4 for 24 and 48 h, respectively.

X-ray Energy Dispersive Spectroscopy (EDS) was used for the qualitative chemical analysis of embryonic shells and statoliths. All specimens were 7- to 8-day-old embryos that had been quick-frozen in liquid nitrogen, while still encapsulated within the egg strand, and stored at −80°C. Two different methods were used for preparing sample material. One procedure included thawing the frozen strands in artificial seawater (+8 ppm strontium) before dissecting for mineralized tissues. Excised stones and shells were then placed in a large drop of distilled water on Formvar-coated 200 hexagonal mesh nickel grids, rinsed by wicking away water, evaporated to dryness, and carbon-coated. In a second procedure we placed the frozen strand in 100% ethanol for five minutes before transferring to a spot dish filled with hexamethyldisilazane (HMDS). After an additional five minutes, the strand was removed from the HMDS, air dried, and transferred to an aluminum stub with double-sided sticky tape. Strands were then broken open with forceps and larvae were arranged on the tape. This material was then carbon-coated.

Analyses were performed on either a Philips-400T transmission electron microscope equipped with STEM and an EDAX spectrometer or a JEOL JSM-840 scanning electron microscope equipped with a Tracor Northern X-ray spectrometer. Shells and stones were examined at 15–40 kV for 500–1000 live seconds. Light micrographs of freshly narcotized material were taken with a Zeiss Photomicroscope III, equipped with polarizing optics.

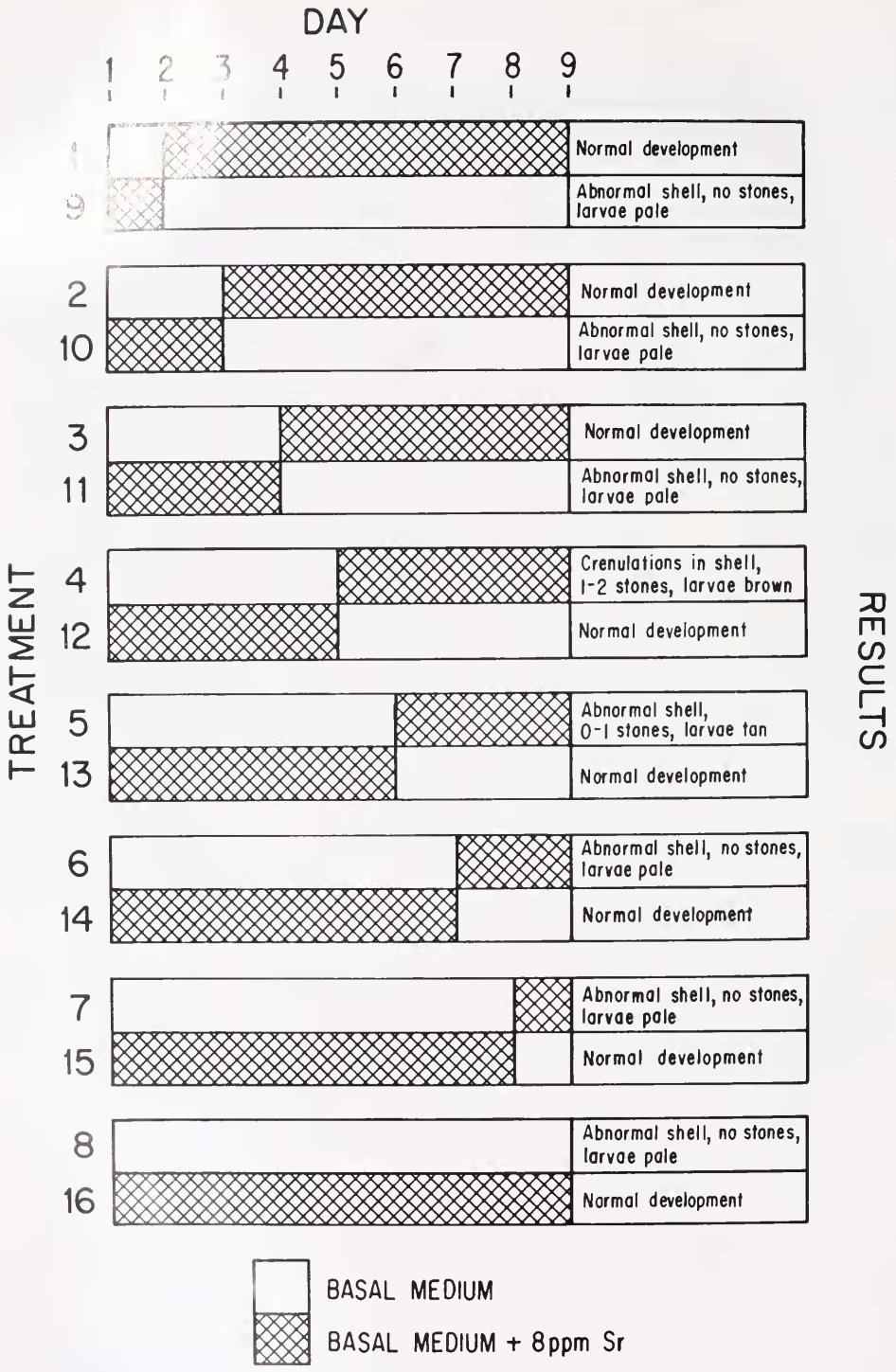

FIGURE 1. Graphical representation and results of sixteen pulse treatments from first pulse experiment. All treatments extended through eight days of embryonic development. Each petri dish generated its own control because 5 of 10 egg strands remained in initial conditions. A natural seawater control is not shown.

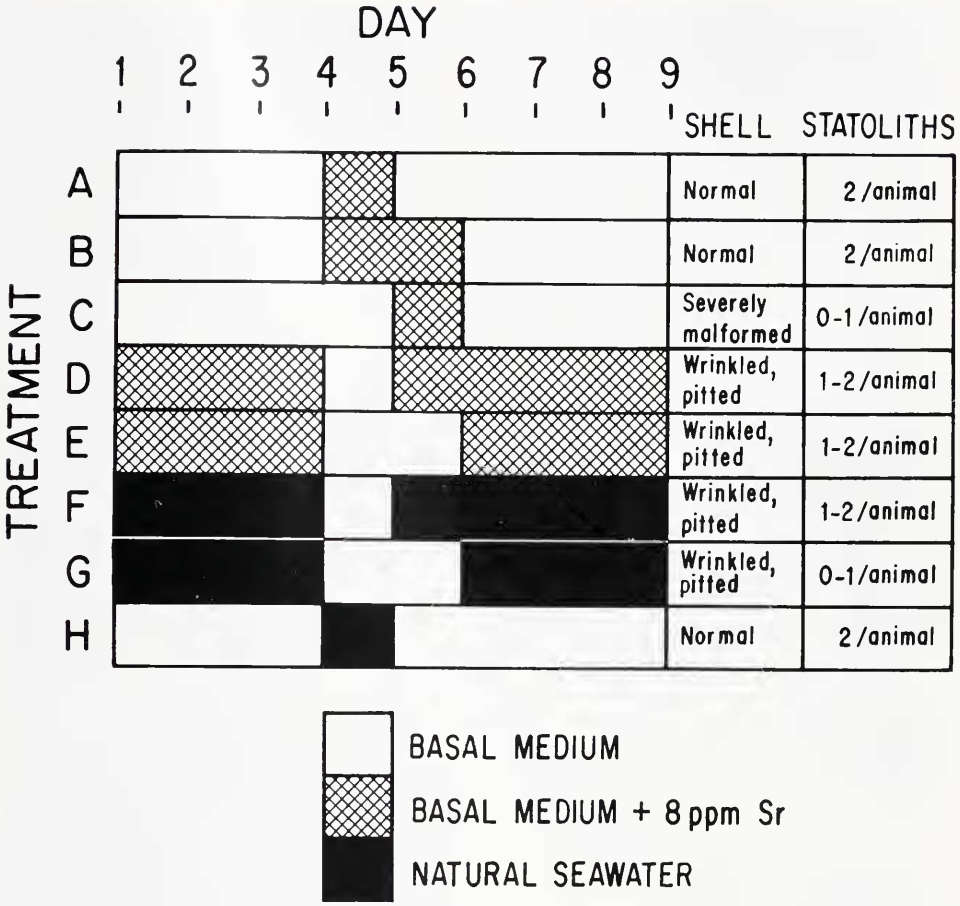

FIGURE 2. Graphical representation and results of treatments from second pulse experiment. All treatments extended through eight days of embryonic development. Controls of basal medium, basal medium plus 8 ppm strontium, and natural seawater are not shown.

Finally, to test the effect of strontium on another mollusc, preliminary experiments were performed on the nudibranch *H. crassicornis.* Embryonic development in this animal is very similar to that of *A. californica.* Fertilized egg strands are deposited by the hermaphroditic adult, and during this 8- to 10-day embryonic period, the shell and statoliths are formed. Single egg strings (about 2 cm in length) were placed in 90 cm × 50 cm crystallizing dishes and filled with 200 mls of test medium (basal medium, basal medium plus 8 ppm strontium, or natural seawater). Cultures were incubated at the optimal temperature of 14°C (Donna McPhie, pers. comm.), and test solutions were changed once daily.

RESULTS

Dose response experiments

Egg strands of *A. californica,* incubated in artificial seawater without strontium, produced larvae with deformed shells and statocysts that lacked the large single statolith

granule. Soft tissue appeared normal; microscopic observations (82×) made throughout development indicated no differences, morphologically or in the time course of development, from the natural seawater controls. Shell and statolith formation were sensitive to concentration differences of one part per million strontium in artificial seawater treatments (Figs. 3, 4). Differences between embryos developing in media with the full complement of strontium (natural seawater and basal medium containing 8 ppm added strontium) and those organisms cultured in the absence of this element first appeared on Day 5 (104 h after oviposition). In the strontium-free artificial seawater, the shell cap was irregular in shape and the single statolith granule was missing from both statocysts. The appearance of the velum and foot (soft tissues) were indistinguishable from the natural seawater controls. Even the empty statocyst cavities

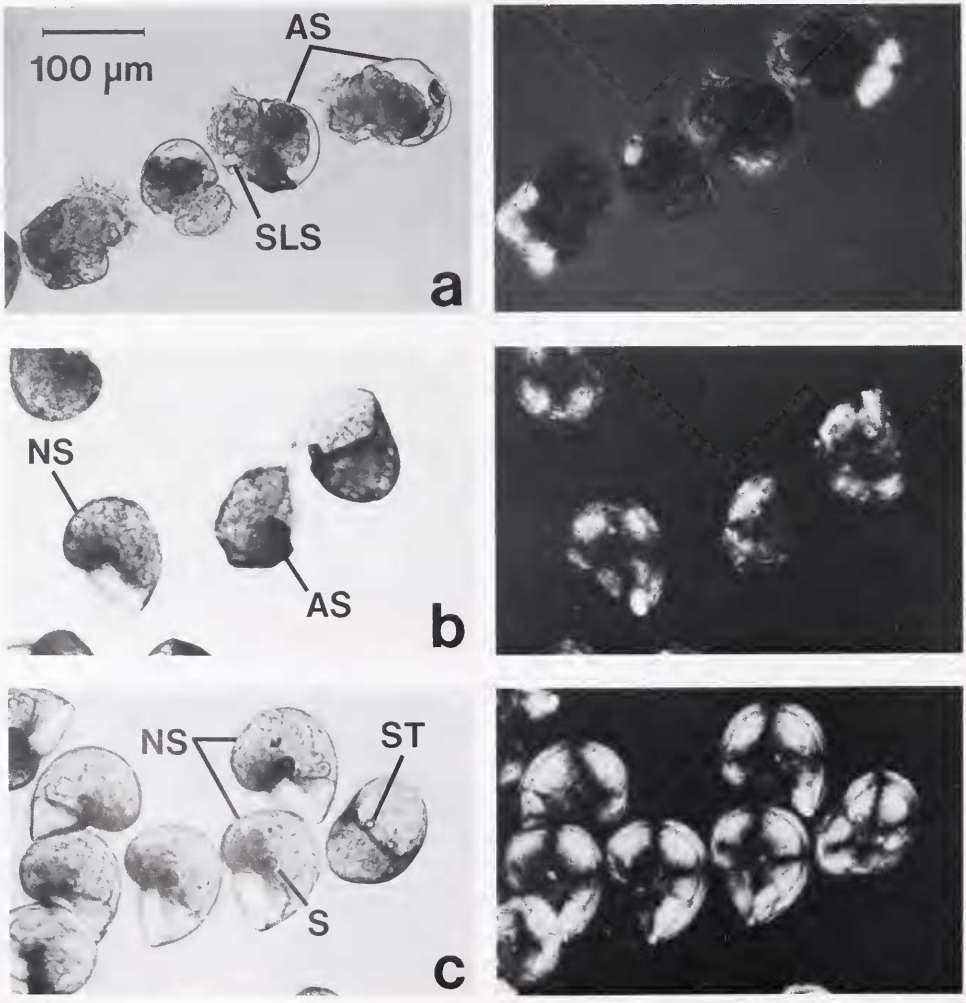

FIGURE 3. Bright field and polarized light micrographs of hatched larvae incubated in artificial seawater during embryonic development at three different strontium concentrations: (a) 1 ppm; (b) 3 ppm; and (c) 7 ppm. Abbreviations: (AS) abnormal shell; (NS) normal shell; (S) statocyst; (SLS) statocyst lacking statolith granule; (ST) statolith granule.

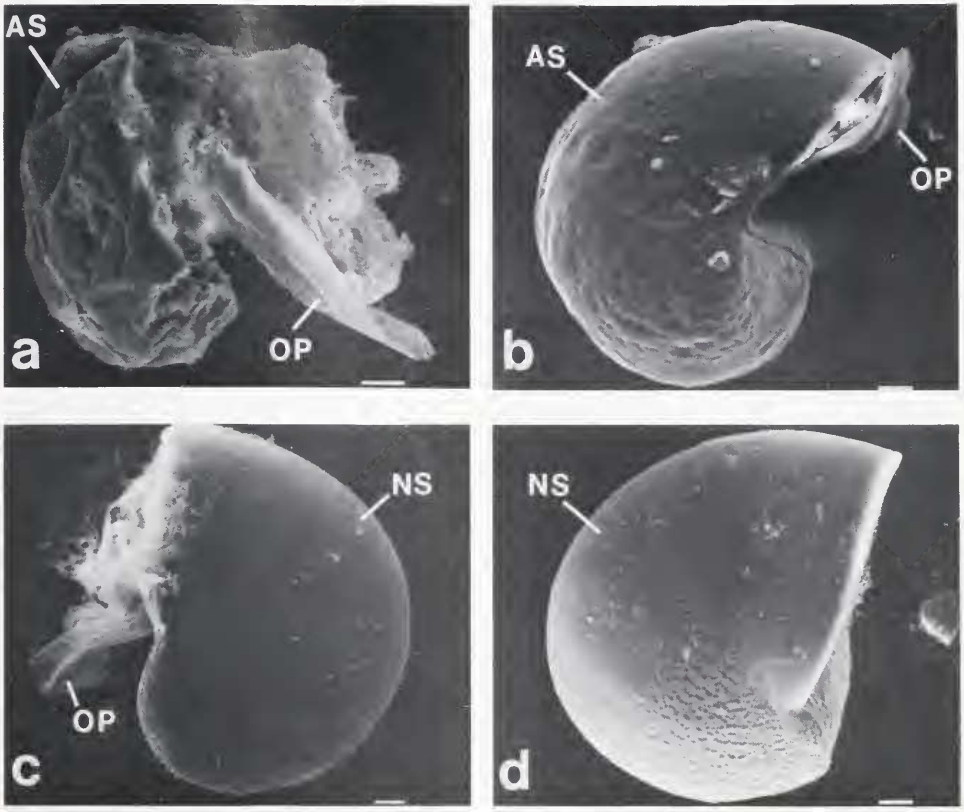

FIGURE 4. Scanning electron micrographs of 8-day-old *Aplysia californica* embryos incubated in artificial seawater at different strontium concentrations: (a) 0 ppm; (b) 3 ppm; (c) 8 ppm; and (d) natural seawater. Abbreviations: (AS) abnormal shell; (NS) normal shell; (OP) operculum. (Scale = 10 μm.)

appeared normal except for the absence of the stones. In the presence of strontium (8 ppm) the shell cap was well defined and the statoliths could be easily seen.

These differences became more pronounced by the end of Day 5 (118 h). Embryos cultured without added strontium had deformed shells that appeared wrinkled, pitted, and were only weakly birefringent when observed under polarized light. The soft tissue had also retracted from the malformed shell.

Another difference noted on Day 5, that became more apparent as development progressed, was the color of the embryonic shell. Darkening of this shell, a brown coloration most evident along the lip and in the whorl, seemed proportional to the amount of strontium in the medium up to 4 ppm. Freshly laid egg strands appear yellow, the color of the fertilized egg visible through the transparent cordon. Those strands cultured in media with 4 ppm or greater added strontium, including natural seawater, appeared to darken from their initial bright yellow color to the normal dark brown observed at the time of hatching. The color of the embryonic shells maintained in the presence of lower concentrations of strontium ranged from a light straw yellow to a darker yellow-tan as the strontium was increased. The empty cordon left behind upon hatching was transparent and colorless after all treatments, further indicating that the color difference was due to color changes in the larvae and not the jelly itself.

All larvae hatched from the egg strands between the normal 200–245 h (8–10 days) after deposition. Veligers from cultures containing 4 ppm or greater added strontium were vigorous swimmers and indistinguishable in their morphological appearance [see Figs. 3(c) and 4(c, d)] and behavior from those cultured in natural seawater. Larvae incubated in artificial seawater containing 0 and 1 ppm added strontium during embryonic development showed the anomalies described above [see Figs. 3(a) and 4(a)]. Also, these larvae moved in an unusually short, rapid, saltatory manner or swam in tight spinning circles; many remained on the bottom of the petri dish, moving along the surface by rocking back and forth on their deformed shells.

A gradation of these effects could be discerned in cultures containing concentrations of 2 and 3 ppm added strontium. In these cultures the abnormalities in shell shape were not as marked and the shell material was qualitatively more birefringent under polarized light [Figs. 3(b) and 4(b)]. Statoliths were absent in organisms grown in the medium with 2 ppm added strontium, but larvae from the medium with 3 ppm added strontium displayed various degrees of the deficiency. We observed animals with no statoliths and others with one or both stones. The shell deformities also varied but as in the 2 ppm treatment, were not as severe as those obtained in the 0 ppm and 1 ppm regimens. The subtleties of this animal's sensitivity to the differences in strontium levels in the external medium can be seen in the scanning electron micrographs of Figure 4.

The anomalies produced in strontium-free seawater and the other dose treatments were reproducible and affected all organisms in the test samples. Strontium bioassays were performed throughout the year on numerous egg masses from a variety of adults. Egg strands incubated in artificial seawater without added strontium always resulted in larvae with the abnormalities described. Different chemical modifications of the artificial seawater failed to affect embryonic development. For example, the elimination of sodium bromide (another minor constituent of natural seawater) instead of strontium chloride, had no detrimental effect on the animals. Also, the use of the basal medium plus additional salts, a modification of Instant Ocean® (see Materials and Methods), did not compensate for the lack of strontium, and none of these additions proved essential for normal embryonic development.

Pulse experiments

Both pulse experiments indicated the critical period for the strontium requirement of *A. californica* began at Day 4 (72 h after oviposition) and lasted no longer than 24 h (see Figs. 1, 2). In the first pulse experiment (Fig. 1) treatments that produced animals with normal mineralized tissue had only one commonality, embryos were exposed to strontium from Day 4 to Day 5. For example, this 24-h period of exposure to strontium is the only common feature between Treatments 3 and 12, but both regimens produced normal larvae. In contrast, Treatments 11 and 12 are identical except for this critical period, yet larvae from the former (exposure to strontium through *Day 3*) resembled those of the basal medium controls, whereas animals from the latter (exposure to strontium through *Day 4*) were normal.

The results of the second pulse experiment gave a more dramatic demonstration of this critical period. Figure 2 shows that animals exposed to strontium only during this 24-h period (Treatment A) were normal. These larvae had two statoliths and their shells were well-formed and devoid of crenulations. A comparison of the scanning electron micrographs in Figures 4(c, d) and 5(a) demonstrates that larvae from Treatment A were indistinguishable from animals reared in artificial seawater plus 8 ppm strontium or natural seawater throughout embryonic development. Identical results

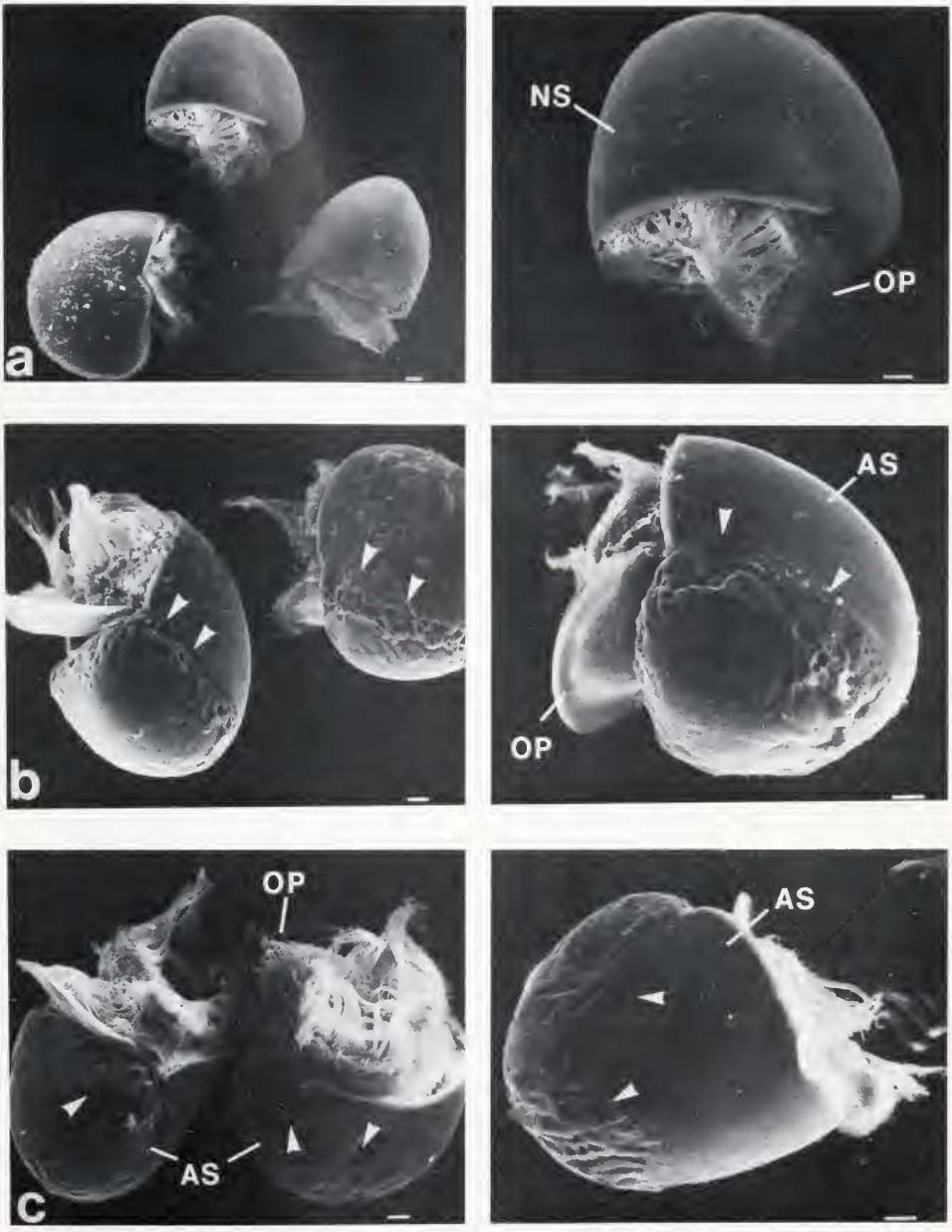

FIGURE 5. Scanning electron micrographs of 8-day-old *Aplysia californica* embryos from second pulse experiment: (a) Treatment A, embryos exposed to 8 ppm strontium for 24 h, Day 4 to Day 5; (b) Treatment D, embryos exposed to 8 ppm strontium throughout development except 0 ppm for 24-h period between Day 4 to 5; (c) Treatment G, embryos exposed to natural seawater throughout development except 0 ppm strontium for 48 h between Days 4 and 6. Abbreviations: (AS) abnormal shell; (NS) normal shell; (OP) operculum; arrows indicate areas of shell deformities. (Scale = 10 μm.)

were obtained with larvae reared in basal medium and exposed to natural seawater during the critical period (Treatment H, Figure 2).

Larvae that had been pulsed with strontium for the 24 h immediately following the critical period, *i.e.*, from Day 5 to Day 6 (Treatment C, Figure 2), were abnormal. These larvae had only one stone when stones were present at all and severely malformed shells. Also the color of the embryonic shells in this treatment was a very light brown whereas the color of the shells in Treatments A and H were indistinguishable from natural seawater or artificial seawater plus strontium controls. In fact, Treatment C was the only regimen to show abnormal shell coloration in the second pulse experiment.

Figure 2 also shows that embryos cultured in media with strontium (artificial seawater plus 8 ppm strontium or natural seawater) and pulsed with basal medium during the critical period resulted in larvae with deficits. Embryos from Treatment D were pulsed with basal medium from Day 4 to Day 5 and grown in artificial seawater with strontium throughout the rest of the embryonic phase. Many animals from this regimen were missing one statolith and the shells were wrinkled and pitted. Scanning electron micrographs [Fig. 5(b)] show the shell abnormalities to be localized in the whorl, that part of the shell formed first. Organisms from Treatment G were pulsed with basal medium for 48 h (Day 4 to Day 6) but otherwise incubated in natural seawater (Fig. 2). Abnormalities were more severe, normal statolith formation appeared to be completely absent with 0–1 stone per animal (a few were found with two small malformed statoliths). The shell defects were more extensive than in Treatment D though again were most evident in the whorl [see Figure 5(b, c)].

The pulse experiments also revealed that deficits induced by strontium deprivation were only marginally reversible. The severity of the abnormalities progressively worsened as exposure to strontium was postponed past the critical window. Exposure before the critical period had no effect on statolith or shell formation. Treatments 9, 10, and 11 from Figure 1 indicate that exposure to strontium before Day 4 did nothing to aid mineralization. All larvae from these regimens resembled those organisms from basal medium controls, having severe shell abnormalities and no statoliths.

Treatments 4 and 5 (Fig. 1) demonstrated that exposure to strontium soon after the critical period *and* extending throughout the remainder of the embryonic period resulted in larvae with less severe abnormalities than those obtained in 0 ppm strontium controls. Larvae from Treatment 4 (exposure to strontium from Day 5) resembled those observed in Treatment D from the second pulse experiment [see Figs. 2, 5(b)]. Crenulations were limited to the shell whorl and a number of larvae were missing one stone. The abnormalities were more severe in Treatment 5 (exposure to strontium from Day 6, see Fig. 1). The vast majority of these larvae lacked stones and the shell margins were more irregular as compared to those obtained in Treatment 4. Finally animals exposed to strontium only after Day 6 (Treatments 6, 7, and 8) resembled those cultured in basal medium throughout the entire experiment.

EDS indicated that mineralized tissues (statoliths and shells) of embryos from all experimental treatments were primarily calcium (as $CaCO_3$). A weak strontium signal could be detected only in treatments that produced normal larvae (*e.g.*, 5 ppm and 8 ppm added strontium). The strontium content of these materials was less than 1% (see Fig. 6), and we were unable to determine if concentrations of shells or stones varied with medium levels. We are now pursuing more quantitative chemical analyses through the use of graphite furnace atomic absorption spectroscopy.

H. crassicornis *experiments*

Preliminary experiments with the nudibranch *H. crassicornis* showed that this animal also required the presence of strontium in defined media for embryonic shell

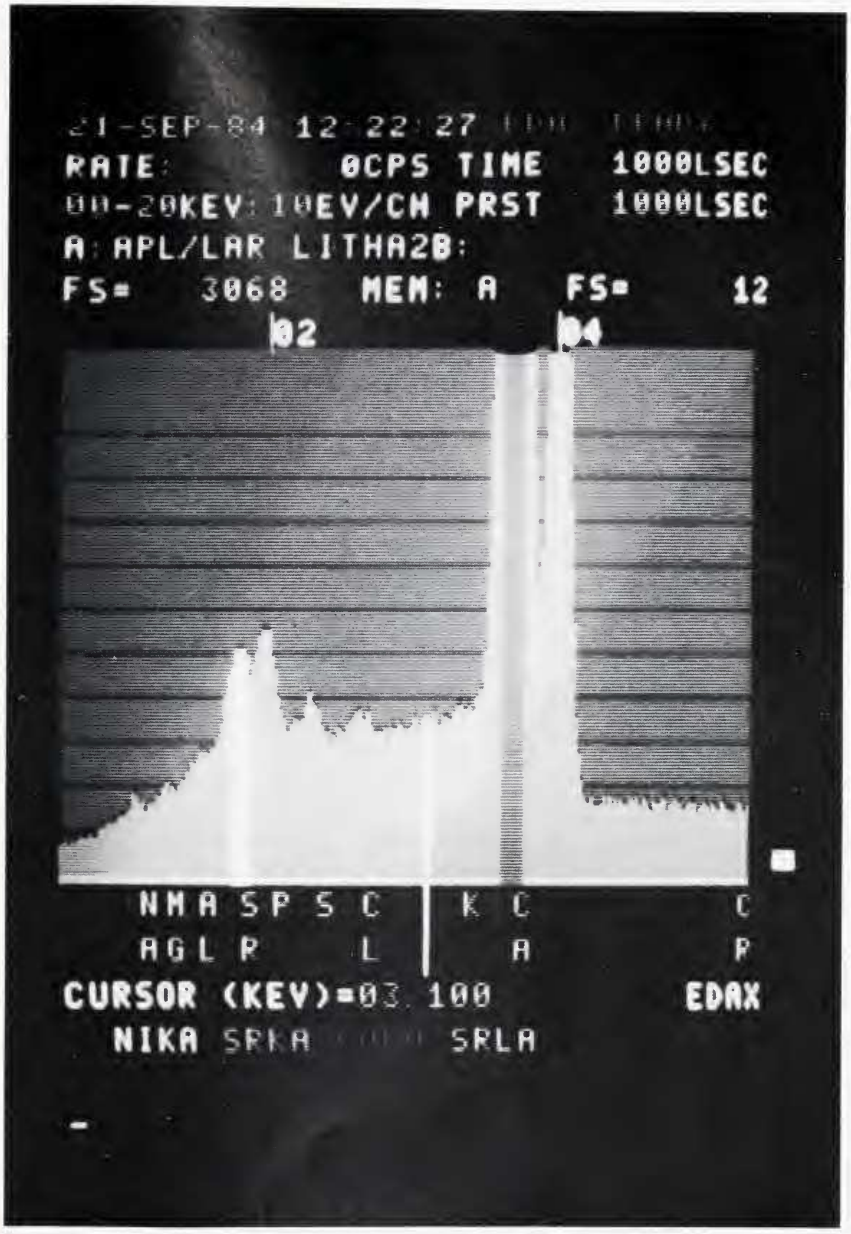

FIGURE 6. Portion of an expanded X-ray energy spectrum (EDS) of a representative statolith from *Aplysia californica* embryo reared in artificial seawater containing 8 ppm strontium. Spectrum obtained with STEM and EDAX spectrometer (20 kV; 1000 live seconds). Data indicates calcium: strontium ratio within the stone approximates that of seawater, *i.e.*, 100:1.

and statolith formation and that the absence of this element induced nearly identical anomalies as described for *A. californica*. Figure 7 shows that deficits appeared to be localized to the mineralized tissue, and we noticed that deformed larvae swam erratically

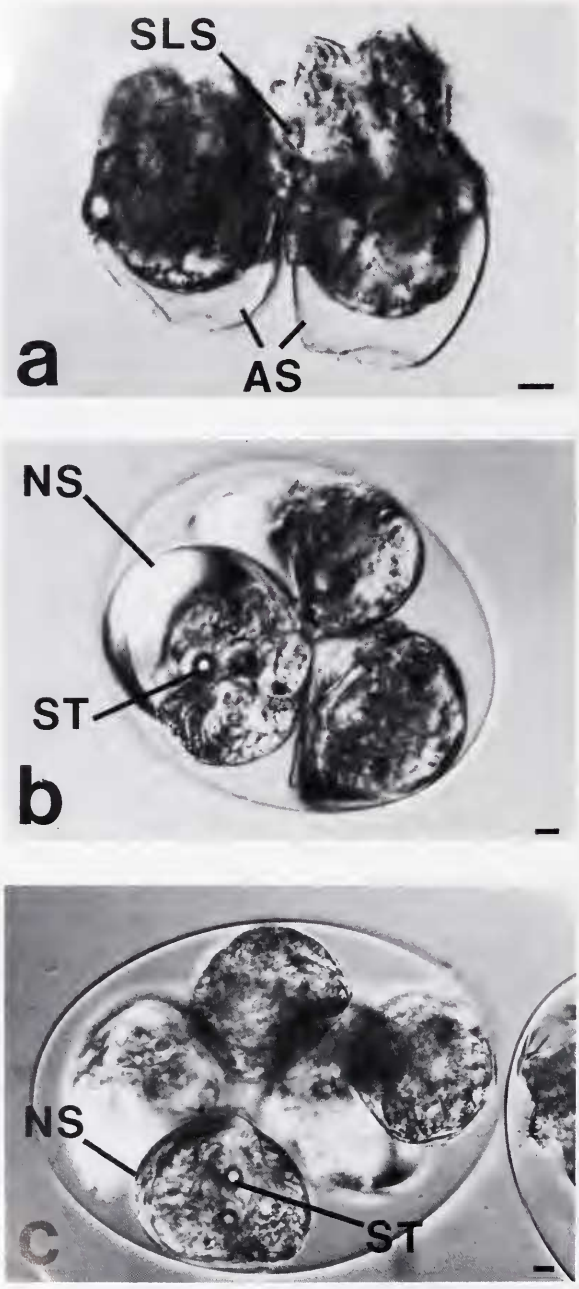

FIGURE 7. Bright field micrographs of 8-day-old *Hermissenda crassicornis* embryos incubated in media with different strontium concentrations: (a) 0 ppm; (b) 8 ppm; and (c) natural seawater. Abbreviations: (AS) abnormal shell; (NS) normal shell; (SLS) statocyst lacking statolith granule; (ST) statolith granule. (Scale = 10 μm.) Note, animals in (b) and (c) are still in egg capsules.

through the water column. The color of the embryonic shell does not normally change during development in this species, and we found that no color changes of any kind were induced by the presence or absence of strontium in the medium.

Discussion

Our study suggests a biological requirement for strontium and indicates a physiological role for this element. Until now strontium has been considered a minor contaminent of both living and fossil tissue (Kinsman, 1969; Riley and Segar, 1970; Binyon, 1978) or as a substitute or tracer for calcium in biochemical studies (Baker and Singh, 1982; Simkiss, 1983). The lack of interest in strontium as a biologically important element *per se* is not surprising since the only organisms with an obvious requirement are the marine protozoa *Acantharia* spp., which possess a test composed of strontium sulfate. Little else is known about this unique exoskeleton since ontogenetic studies establishing specific chemical requirements or the molecular mechanism for its formation have not been reported (see Anderson, 1981).

It appears that strontium is essential to the mineralization process in embryonic *A. californica.* To date, no one has directly addressed the possible role of trace metals in shell deposition, and there has been little attempt at a synthesis of the vast amount of information on factors that affect the trace metal content of shells and the mineralization process itself (see Wilbur, 1972; Simkiss, 1976; Wilbur and Saleuddin, 1983). The strontium content of mollusc shells is low, but differences in shell strontium content usually have been interpreted as a measure of an animal's ability to exclude it from the calcium carbonate-organic matrix (Dodd, 1967). In fact, it has been suggested that the higher the phyletic level of the organism the greater its ability to exclude "foreign ions," such as strontium and magnesium, from the skeleton (Dodd, 1967).

Our results suggest that embryos of *A. californica* take up strontium from seawater and use it for structural and sensory development. Deprivation of strontium during this early stage results in an animal with morphological and behavioral deficits. The morphological deficits include the malformed shell and its abnormal color development. Darkening of the larvae within the egg strand, from yellow to brown, and its dependence on strontium concentration reflects the extent of shell mineralization occurring within the egg capsules. Kriegstein (1977) also noted that the lip of the shell in newly hatched veligers is characteristically golden-brown. Therefore, a possible explanation is that the dark coloration arises from the organic material or matrix (Wilbur, 1972) laid down during mineralization which is visible through the transparent cordon. Because shell formation is dependent on the strontium concentration of the seawater, so then is the apparent color development of the larvae within the egg strand. Finally, the other deficit is the absence of the statoliths. The spinning behavior, induced by strontium deprivation, can be explained by the absence of statoliths whose formation appears to require this element at the beginning of Day 4 (72 h after oviposition). The invertebrate statocysts are balance receptors (Coggeshall, 1969; for review see Markl, 1974) and provide a directional sense through the interaction of the stone with the sensory cilia of the hair cells (Colmers, 1977; Budelmann, 1979; Stommel *et al.,* 1980). In *Aplysia* spp. they coordinate locomotion and swimming (Kandel, 1979), and we find that affected larvae move erratically upon hatching.

Although structural defects of the shell may contribute to spinning, the lack of statoliths and subsequent loss of sensory input is sufficient to explain the behavioral disorder. Our experience indicates that the deformed shell does little to inhibit movement. For example, in our culture work, we have observed larvae with malformed shells and fully developed statoliths that swim normally; also, larvae that have left

their shells do not spin. A similar spinning syndrome has been reported in a number of cephalopod larvae which have only rudimentary shells (Colmers *et al.,* 1984). "Spinner" cephalopod larvae (three species of squid and three of octopus) swim erratically upon hatching in recirculating systems of artificial seawater. The only morphological deficit in these animals is the missing statolith granule in both statocysts. Another example of abnormal statolith development affecting behavior is that reported by Crow and Harrigan (1979) in the nudibranch *H. crassicornis.* Animals raised in the laboratory developed statocysts with a single statoconium as compared to normal statocyst cavities filled with many statoconia. The authors suggested that this morphological difference between laboratory-reared and field-collected adult *H. crassicornis* explained some of the reduced variability in the response to light of the laboratory-reared animals.

The required presence of strontium in artificial seawater during embryonic development and the distinct consequences of its absence is not limited to *A. californica.* In fact, each mollusc tested under the conditions described in this paper has demonstrated a requirement for this element during embryonic mineralization with nearly identical symptoms of the deficiency. As described above, larva of the nudibranch *H. crassicornis* have malformed shells and lack statoliths when incubated in basal medium as embryos. Also, collaborative studies with Roger Hanlon of the Marine Biomedical Institute in Galveston have demonstrated that strontium is required for statolith formation in two species of squid, *Loligo pealei* Lesueur, 1821, and *L. vulgaris* Lamarck, 1799. Embryos incubated in basal medium became squid larvae with soft tissue that appeared normal but with empty statocyst cavities, *i.e.,* "spinners." The addition of strontium to the incubation medium resulted in larvae with normal stones (Hanlon *et al.,* in prep.).

Finally, we have determined that the shipworm *Bankia gouldi* (Bartsch, 1908) (Class Bivalvia) also appears to require strontium. These animals are oviporous broadcast spawners that undergo embryonic development in the 24-h period immediately following fertilization. During this stage of development, the hinged prodisoconch I is formed; the statoliths, however, develop about 18 days later during the larval phase. The absence of strontium from the incubation medium during the first 24 h prevented shell formation, while again the soft tissues appeared to proceed through normal development. The shell-less animals were vigorous swimmers. The addition of strontium resulted in normal larvae (Gallager and Bidwell, in prep.).

The molecular mechanism for producing the behavioral and structural deficits observed in *A. californica* is not yet known, but the pathogenesis seems clear. The shell and stones are primarily calcium carbonate (Coggeshall, 1969) a crystal that does not require strontium for its formation *in vitro.* Yet crystal formation *in vivo* is very sensitive to the strontium chemistry of the external medium. Pulse and dose response experiments demonstrate that the shell crystal is a sensitive visual record of the chemical history with respect to the amount of strontium in the medium and the time of exposure. Also it appears that this organism has little ability to store this element before the critical period of Day 4, the onset of mineralization.

How then is strontium used in the formation of skeletal carbonate and what is the significance of the critical window of exposure? Perhaps this element is a cofactor of an enzyme involved in initiating mineralization during the formation of the shell cap and the single statoconium, though to date, no strontium metalloenzyme has been characterized. Three enzymes that have been implicated in the biomineralization process are carbonic anhydrase, alkaline phosphatase, and the ATPases (Simkiss, 1976; Wilbur and Saleuddin, 1983). Alkaline phosphatase is an attractive candidate. Though little is known about its role in mineralization, it has been suggested that it may be

involved in removing crystal poisons (Simkiss, 1964) or synthesizing matrix material (Bradfield, 1950; Kroon, 1952). The metallo-chemistry of this large and ubiquitous family of enzymes is suggestive of a place for strontium. It is generally agreed that zinc and an alkaline earth metal such as magnesium or calcium are an integral part of most of these proteins (McComb *et al.*, 1979). Also, strontium can partially restore the activity of a calcium containing alkaline phosphatase from *Micrococcus sodenensis* (Glew and Heath, 1971). The action of strontium in *A. californica* is specific since the large amounts of calcium and magnesium present in the medium are unable to substitute for the small amount of strontium essential for mineralization. Also, the presence of a variety of trace metals, boric acid, and inorganic phosphate cannot compensate for the lack of strontium. In fact, we find that the simple basal medium plus strontium has been sufficient for rearing *A. californica* through an F_6 generation.

Our results have important implications for a variety of disciplines including molluscan physiology, neurobiology, and paleoecology. Until now strontium has been viewed in the context of a minor contaminent of mineralized tissue, an element with a similar chemistry to calcium and thus a convenient probe for shell deposition, not as a requirement to a vital pathway of the process itself. Also, paleoecologists have long recognized that if strontium is to be used as an indicator of ancient environments, its physiological and diagenetic activities must be disentangled (Rosenberg, 1980). Until now, however, any understanding of strontium's biological importance has not progressed beyond reporting contents of this element in living and fossil shell material (Rosenberg, 1980). Finally, strontium appears to be essential for the normal development of behavior in *A. californica, Loligo pealei, L. vulgaris,* and *H. crassicornis* through its effect on formation of statoliths.

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